A METHOD FOR THE STUDY OF THE FORMATION OF VOLATILE SULFUR COMPOUNDS BY BACTERIA

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The study of hydrogen sulfide formation by bacteria has long been handicapped by the lack of an accurate method for the quantitative estimation of very small quantities of the gas. The lead acetate paper test may be used where quantitative accuracy is of little importance, but this method is too crude for exact determination. Lead and iron salts have been added directly to the culture media by various investigators (Wilson, 1923) as a qualitative and approximately quantitative test for differentiation purposes.

Recently the determination of hydrogen sulfide by titration with standard iodine solution has been advocated. Heap and Cadness (1924) employed N/100 solutions of iodine, a stream of carbon dioxide being used to carry over the hydrogen sulfide. Fellers, Shostrom, and Clark (1924) recommend N/40 iodine solutions and separation of the hydrogen sulfide by means of a current of air. These methods are open to the objection that they are not sufficiently delicate and are not specific for hydrogen sulfide, as other substances may be oxidized by the iodine (mercaptans).

With a method recently devised (Almy, 1925), hydrogen sulfide in quantities as small as 0.002 mgm. (2 micromilligrams) may be satisfactorily determined. The new procedure has the added advantage that, so far as we have been able to determine, it gives hydrogen sulfide only. The determination is accomplished by aeration of the acidified aqueous suspension or solution of the

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samples with carbon dioxide, absorption of the evolved hydrogen sulfide in dilute zinc acetate solution, and evaluation of the hydrogen sulfide by a measurement of the strength of "methylene blue" color produced upon adding p-amino dimethylaniline hydrochloride, hydrochloric acid, and ferric chloride to the zinc acetate mixture. The original article gives the details of the method.

This methylene blue method was successfully adapted to the study of hydrogen sulfide formation by bacteria in broth cultures. Certain features were considered essential for the proper conduct of such studies. It should be possible to follow the *rate* of formation of hydrogen sulfide without loss of the gas, without changing the conditions of growth of the bacteria and with the oxygen supply under adequate control. Following the rate by withdrawal of aliquot portions of the broth for analysis is obviously of no value, as no account is taken of that not inconsiderable portion of the gas which escapes from the medium. This loss would naturally be greater while the medium was acid than while it was alkaline. Likewise it is not satisfactory to attempt to follow the rate by intermittent aeration of a single broth culture, because not all of the gas would be removed in this manner when the medium is alkaline. Furthermore, the irregular aeration would introduce a variation in the condition of the medium which would not be comparable with the conditions obtaining in ordinary cultures, which usually remain undisturbed during the entire incubation period.

PROCEDURE

Bearing these matters in mind, the following apparatus and procedure were devised. The medium is sterilized in a 2-liter separatory funnel and after cooling to 37.5°C. is inoculated with a suspension of the organism to be studied. After thorough mixing to insure uniform distribution of the bacteria, aliquot portions (100 cc.) of the broth are run into flasks, the portions being accurately measured with a pipette connected to the funnel (fig. 1). Before use, the pipette is sterilized with the inlet and outlet tubes protected by plugged test tubes as shown for the outlet tube.

The test tube protecting the outlet should be replaced between each measurement of the aliquots.

The ordinary Florence flask with neck at least 10 cm. long (fig. 2) is recommended as a culture flask. Two sizes of glass

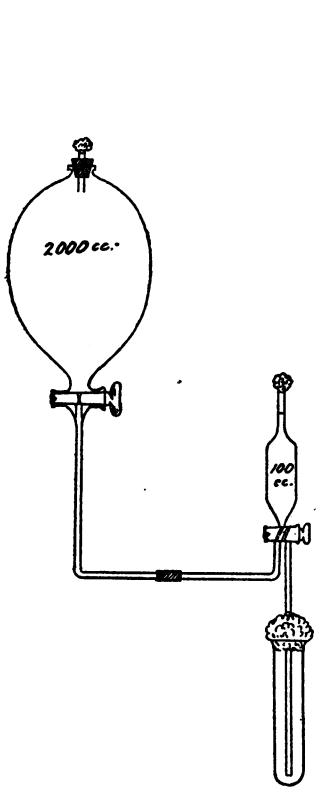

FIG. 1

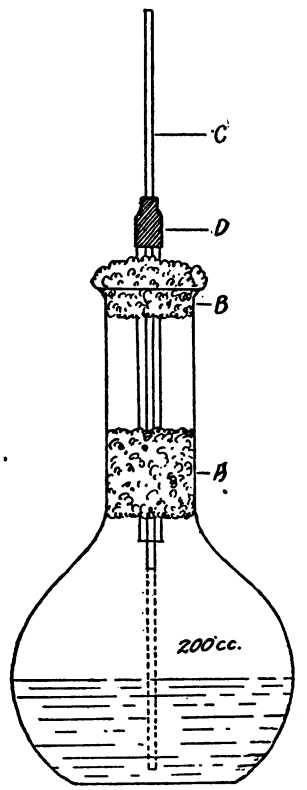

FIG. 2

FIG. 1. METHOD OF MEASURING ALIQUOTS OF INOCULATED BROTH
FIG. 2. CULTURE FLASK

tubing are selected. The smaller tube fits inside the larger (about 5 mm. inside diameter), leaving only a very small annular space. Over a length of about 4 inches near the end of the larger tube (15 cm. long) one layer of cotton twine is tightly wound. A washed rubber band may be used in place of the twine. This

serves to hold in place the plug of absorbent cotton which is wound in even layers (not too thick) over the twine or rubber, forming a neat compact plug, large enough to fit snugly into the lower end of the neck of the flask (*A*, fig. 2). A 4 mm. section of soft rubber tubing about 3 cm. long (*D*, fig. 2) is placed on the other end of this tube for a distance about half the length of the rubber tube. The smaller tube (22 cm. long) is then inserted in the larger, where it is held in place by the short section of rubber tube. A small plug of cotton is inserted in the upper end of the small tube. The plugged flasks are then sterilized. To minimize the possibility of contamination of the double plug device during the subsequent filling of the flask with inoculated broth, it is preferable to sterilize it in a test tube of approximately the same diameter as the neck of the flask, inserting it into the flask immediately after the filling.

The lower of the two plugs is moistened with 5 to 10 cc. of sterile 1 per cent zinc acetate solution as an absorbent for the hydrogen sulfide, the upper plug being withdrawn slightly to admit the slender tip of the pipette. The flask is then placed in the incubator. If desired, the smaller tube (*C*, fig. 2) may then be attached to a gas supply (hydrogen, carbon dioxide, air) with the lower end of the tube above the surface of the broth, or below the surface as shown by the dotted lines. For most purposes, one bubble per second is a satisfactory rate of flow for the gas through the medium. At the pressure thus created the zinc acetate solution in the lower plug should accumulate at its upper surface, providing a layer of solution about $\frac{1}{8}$ inch deep on top of the plug. If appreciable drying out of the lower plug occurs during the incubation period, more zinc acetate solution may be added without danger of contamination.

At the end of the period of incubation tube *C*, after being lifted out of the broth, is disconnected from the gas supply. The tube *C* is then withdrawn and 2 cc. of the broth is removed by inserting the elongated tip of the pipette through the larger tube. Bacterial counts are determined by the plate-dilution method. The rest of the broth and plugs *A* and *B*, after being unwound from the

tube, are placed in the aerating cylinder and hydrogen sulfide is determined by the methylene blue method.

EXPERIMENTAL

Throughout the investigation 3 per cent pepton solution containing 0.5 per cent sodium chloride is employed as the culture medium. The initial hydrogen ion concentration is adjusted to approximately pH 8.0 before sterilization in every case, yielding a sterile solution of approximately pH 7.6. All cultures are incubated at 37°C.

TABLE 1

Hydrogen sulfide produced from peptones by Proteus vulgaris (100,000 per cubic centimeter) in twenty-four hours. Initial pH approximately 7.6

PEPTON	FINAL pH (APPROXIMATE)	FINAL COUNT OF BACTERIA PER CUBIC CENTIMETER	FINAL H_2S	RELATIVE H_2S FORMATION (C = 1)
			mmgm.	
A	7.4	225,000,000	738	24
B	7.6	370,000,000	584	19
C	7.4	240,000,000	31	1
D	7.4	260,000,000	215	7
E	7.4	300,000,000	473	15

Hydrogen sulfide from peptones

Tilley (1923) and others have shown that the quantities of hydrogen sulfide which may be yielded by peptones under the action of bacteria vary. The methods used by previous investigators for the determination of hydrogen sulfide formation were only approximately quantitative. Five brands of peptones, representative of those on the market today, were therefore tested by the new method.

For this experiment, the peptones were sterilized in the 200 cc. flasks in the autoclave for fifteen minutes at 15 pounds pressure. Ordinary plugs were inserted in the flasks for the sterilization, the double plugs being sterilized independently. Suspensions of *Proteus vulgaris*, made from vigorous twenty-four hour slant agar cultures, were so inoculated that the resulting broth contained 100,000 bacteria per cubic centimeter. The flasks were incu-

bated for twenty-four hours with air bubbling throughout them at the rate of about one bubble a second. The results are shown in table 1.

All but one of the peptones gave a large volume of hydrogen sulfide. Peptones D, E, B and A yielded 7, 15, 19 and 24 times as much of the gas as pepton C. Another experiment, a duplicate of the first except that the cultures received an initial inoculation of 1,000,000 bacteria per cubic centimeter, gave practically identical figures for the amount of hydrogen sulfide produced in twenty-four hours, but the final bacterial count was greater in the second experiment than in the first. This indicates that the media were practically exhausted of the readily available material from which the gas was formed. The hydrogen ion concentration did not change appreciably during this short period.

Rate of hydrogen sulfide formation from pepton

It is well known that several hours are required for the growth of bacteria to become sufficiently vigorous to produce enough of the end products to give a positive test. The length of this "fore-period" would depend upon several factors, but under closely prescribed conditions it might be predicted to be solely a characteristic of the organism. Using a popular brand of pepton of American manufacture (pepton E), the rate of formation of hydrogen sulfide by *Salmonella aertrycke* and *Proteus vulgaris* was studied. Results typical of those obtained in several experiments are shown in tables 2 and 3.

The rate of hydrogen sulfide production is not uniform in the case of either of the bacteria. A sudden rise in hydrogen sulfide formation in the case of *S. aertrycke*, came some 30 minutes after an apparent rapid increase in microörganisms. At the period when the gas was first consistently detectable in the media, in the case of either culture, it was not possible to make observations fast enough to obtain the intermediate values which would show the initial slope of the rate curve. The maximum hydrogen sulfide production by *S. aertrycke* was about half that of *Proteus vulgaris*. *S. aertrycke* produced the maximum volume of gas in $6\frac{1}{4}$ hours. The other organism slowly produced hydrogen sul-

fide from the 13½ hour period to the 52-hour period, when the value was about 65 per cent higher than at the 13½-hour period.

From these data it may be tentatively concluded that when the bacteria have reached the stage of rapid multiplication they quickly attack the readily decomposable sulfur compounds

TABLE 2

Hydrogen sulfide produced from Pepton E by S. aertrycke. Culture not aerated

INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
hours		*mmgm.*
0	270,000	0
4½	109,000,000	0
4¾	14,000,000	8
5	1,400,000	0
5¼	31,000,000	Trace
5¾	117,000,000	0
6¼	190,000,000	202
7	267,000,000	202
8	158,000,000	196
9	245,000,000	196
9½	460,000,000	178
27	15,000,000	199
53	365,000	

TABLE 3

Hydrogen sulfide produced from Pepton E by Proteus vulgaris. Culture not aerated

INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
hours		*mmgm.*
0	1,000,000	0
8¾		0
9½	190,000,000	Trace
13½	400,000,000	251
27½	600,000,000	267
31½	5,000,000,000	304
52	720,000,000	413

present in the pepton, with the formation of hydrogen sulfide. Any further hydrogen sulfide formation depends upon the ability of the organism to split the residual complex. Apparently *Proteus vulgaris* possesses the power to decompose polypeptides in greater degree than *S. aertrycke.*

Influence of oxygen on hydrogen sulfide formation

Some interesting data were obtained on the effect of oxygen on *Proteus vulgaris* when grown in a solution of Pepton E (table 4).

Under the conditions of the test *Proteus vulgaris* grew better and produced hydrogen sulfide faster in the presence of oxygen than in its absence. The cultures which were not aerated followed a middle course between those aerated with hydrogen and those aerated with air. Similar results were obtained in other

TABLE 4

Effect of air and hydrogen on hydrogen sulfide production from Pepton E by Proteus vulgaris

METHOD OF INCUBATION	TIME OF INCUBATION	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
	hours		*mmgm.*
Not aerated	0	4,000,000	0
	5	24,000,000	181
	7	160,000,000	214
	27	150,000,000	434
	53	60,000,000	434
Aerated with air	0	4,000,000	0
	5	280,000,000	239
	7	500,000,000	355
	27	1,300,000,000	428
	53	3,100,000,000	420
Aerated with hydrogen	0	4,000,000	0
	5	130,000,000	217
	7	140,000,000	277
	27	20,000,000	336
	53	30,000,000	424

tests of like nature. Apparently decomposition of food products by this organism proceeds more quickly when the products are exposed to the air than when they are protected from it.

Hydrogen sulfide from cystin

Previous experiments had indicated that *S. aertrycke* and *Proteus vulgaris* quickly produced hydrogen sulfide from the readily decomposable sulfur compounds of the pepton. It was

believed that preformed cystin comprises the greater part of the mother substances from which the gas is produced. If this is true the organisms should promptly decompose all of any cystin added to the culture medium and yield hydrogen sulfide from it in practically theoretical quantities. Accordingly *Proteus vulgaris* was allowed to grow in a solution of pepton E with and without the addition of the amino-acid cystin. Five cubic centimeters of sterile 0.2 per cent cystin dissolved in very weak hydrochloric acid solution (0.2 gram cystin + 20 cc. 0.075 N hydrochloric acid + water to 100 cc.) was added to 100 cc. of the sterile pepton solution in the usual 200 cc. flask. After inoculation, the medium

TABLE 5

Hydrogen sulfide produced from cystin by Proteus vulgaris

METHOD OF TREATMENT	INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	H_2S
	hours		*mmgm.*
0.01 gram cystin added; culture aerated	0	1,000,000	0
	2	14,000,000	0
	4	53,000,000	3.5
	5	360,000,000	6.8
	7	850,000,000	2,488
	25	1,030,000,000	3,310
	50	3,000,000,000	3,310
No cystin; culture not aerated	50	81,000,000	461
No cystin; culture aerated	50	3,200,000,000	461

was incubated at 37°C. and analyzed at intervals up to fifty hours total incubation period. The cultures, except in the case of one of the controls, were aerated at the rate of about two bubbles of air a second.

The results (table 5) show that a slow bubbling of air is beneficial to the growth of *Proteus vulgaris*. The amount of hydrogen sulfide produced in fifty hours from broth without added cystin, whether aerated or not, however, was the same. In the growth with added cystin about 75 per cent of the total hydrogen sulfide obtained in fifty hours was produced between the fifth and seventh hours of incubation. Analysis showed that the cystin employed in the experiment contained 25.9 per cent sulfur (theory for chemi-

cally pure cystine, 26.67 per cent sulfur). Subtracting the weight of gas formed in the absence of cystin from that formed in its presence gives a value (2849 mmgm.) which agrees well with the quantity of hydrogen sulfide (2752 mmgm.) theoretically obtainable from the 0.01 gram of cystin. Considering the nature of the experiment, this agreement is very close and is believed to prove that all of the added cystin was decomposed.

TABLE 6

Hydrogen sulfide produced from pepton E by bacteria. Cultures aerated with air

BACTERIUM	HYDROGEN SULFIDE AFTER TWENTY-FOUR HOURS
	mmgm.
S. aertrycke	350
Esch. coli (variety *communior*)	15
Esch. coli (a)	462
Proteus vulgaris X19 (a)	60
Proteus vulgaris (a)	462
Proteus vulgaris (b)	458
Proteus vulgaris (c)	462

(a) Procured from Bacteriological Laboratory, University of Iowa, Iowa City, Iowa.

(b) Procured from Bacteriological Laboratory, Ohio State University, Columbus, Ohio.

(c) Bureau of Chemistry strain.

Hydrogen sulfide produced by different strains of bacteria

Numerous tests of the hydrogen sulfide producing power of different bacteria have been reported in the literature. In expressing the results the attempt was made to indicate an approximate quantitative relationship between the amounts of gas produced in a given time. Tilley (1923a) reports a variability in the amount of hydrogen sulfide produced by different bacteria in a beef infusion agar medium containing added cystin. He records the quantities in relative terms, as "small amount," "moderate amount," and "large amount." Similar variations were noted by Sasaki and Otsuka (1912), using Fränkel's solution, and by Myers (1920) and others. Apparently bacteria which can pro-

duce hydrogen sulfide in suitable media differ in the speed of their action, a difference probably related in part to the rate of growth. It was considered of interest to test various strains of *Proteus vulgaris* to determine whether the amount of hydrogen sulfide produced from pepton E, in the case of this organism, was a species characteristic or varied with the strain, and also to test the ability of two or three other organisms to produce the gas under the same conditions.

The organisms used are listed in table 6. The broths were inoculated with 1,000,000 bacteria per cubic centimeter, no counts being made on the cultures at the end of the twenty-four-hour period except in the case of *Proteus vulgaris* (c), which showed 2,000,000,000 per cubic centimeter. Three strains of *Proteus vulgaris* produced practically the same amount of hydrogen sulfide in twenty-four hours. The remaining strain, *Proteus vulgaris* X19, differing culturally from the other strains only in that it was somewhat erratic, produced about one-eighth as much. Two strains of *Esch. coli* differed widely in hydrogen sulfide formation, *S. aertrycke* gave lower results than the strains of *Proteus vulgaris* but higher results than when the culture was not aerated (table 2).

Production of mercaptans by bacteria

The odor of mercaptans may be detected in aqueous solutions when they are in concentrations as low as one part in a million (Watson, 1922), but as yet satisfactory quantitative methods for use in this range are not available. The most delicate qualitative test for mercaptans involves the use of isatin in concentrated sulfuric acid, but the reagent does not lend itself readily to the quantitative determination of this class of sulfur compounds.

The new method of analysis for hydrogen sulfide in bacterial cultures adapts itself admirably to the simultaneous determination of other reducing substances. Kimball, Kramer, and Reid (1921) have shown that mercaptans may be accurately estimated by titration with iodin. By placing on the end of the aeration train of the hydrogen sulfide apparatus a series of four additional bottles, the first being a gas trap, the second containing about 75

cc. of N/100 iodin, the third a measured quantity of N/100 thiosulfate solution appropriately diluted with water, and the fourth a weak starch solution, it is possible to measure the amounts of reducing substances, other than hydrogen sulfide, which are evolved from the medium. Mercaptans and thioethers, under these circumstances, would pass through the zinc acetate solution and be acted upon by the iodin. The mercaptans change to stable alkyl disulfides and the thioethers form iodin addition compounds which do not affect the iodin titre as the iodin is quantitatively liberated therefrom during the back titration with sodium thiosulfate solution. It is thus seen that the method is practically specific for mercaptans. The titration is carried out in a manner similar to that used by Fellers, Shostrom, and Clark (1924) for the determination of hydrogen sulfide. It is recognized that the suggested procedure has the disadvantage that it is limited in delicacy by the strength (N/100) of the thiosulfate solution required for the back titration. The efficacy of the method was tested by analyzing solutions containing known quantities of ethyl mercaptan. Duplicate determinations checked very closely, 98.3 per cent of the mercaptan being accounted for.

Repeated trials demonstrated that detectable quantities of mercaptans were not formed by *Proteus vulgaris*, *S. aertrycke*, or *Esch. coli* in a solution of pepton E when incubated for two days at 37°C. without aeration. It is believed, however, that interesting information on mercaptan formation by strict anaerobes could be obtained by this method in view of the observation by Rettger (1906) that mercaptans are formed in abundance by anaerobic organisms in pure culture.

SUMMARY

A method for following the rate of hydrogen sulfide production by bacteria in broth cultures recently devised in the Bureau of Chemistry may be successfully applied under proper conditions to the study of aerobes, facultative anaerobes, and strict anaerobes.

The ratios of the amounts of hydrogen sulfide produced by

Proteus vulgaris in twenty-four hours from five peptones on the market were found to be 1:7:15:19:24.

A large proportion of the total hydrogen sulfide yielded by *Proteus vulgaris* and *S. aertrycke* from pepton in two days is produced in a comparatively short time in the early part of the incubation period.

Proteus vulgaris grew better and produced hydrogen sulfide faster from pepton under aerobic conditions than in the absence of oxygen.

Cystin gave practically the theoretical amount of hydrogen sulfide in pepton inoculated with *Proteus vulgaris*.

Three out of four authentic strains of *Proteus vulgaris* gave the same amount of hydrogen sulfide from pepton in twenty-four hours.

Tests for the formation of mercaptans by *Proteus vulgaris*, *S. aertrycke*, or *Esch. coli* from pepton during a two-day incubation were negative.

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